Modified Rapid DNA Extraction Protocol for High Throughput Microsatellite Analysis in Wheat

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ABSTRACT

New technology is allowing marker-assisted selection to fulfill the promise of increasing efficiency of cultivar development. However, these techniques depend upon the ability to extract DNA from large populations of plants. The objective of this project was to develop a high-throughput DNA extraction procedure without the need for greenhouse space or growing wheat (*Triticum aestivum* L.) plants. A sodium hydroxide rapid DNA extraction was modified for a 96-well format to reduce costs. Seeds were germinated in 8-well tissue culture plates, and 4-d-old seedling tissue was used to extract DNA by means of sodium hydroxide methodology. Approximately 1 μg of genomic DNA per 10 mg of tissue was isolated at a cost of about $0.10. The DNA quality was verified by amplification of microsatellite markers. Results were consistent with either fresh or stored tissue extracts. This technique allows one person to extract nearly 1000 storage-stable DNA samples daily, while keeping costs at a minimum.

MARKER-ASSISTED SELECTION in plant breeding programs is limited by the availability of markers, ability to extract DNA from large populations of plants, and, most importantly, high cost. New technologies, such as polymerase chain reaction (PCR), capillary gel electrophoresis, and robotic automation, have evolved to address the limitations faced by plant breeders. The potential of utilization of marker-assisted selection has increased as more molecular markers have been identified. In wheat alone, over 400 microsatellite or simple sequence repeat (SSR) markers have been reported (Bryan et al., 1997; Devos et al., 1995; Plaschke et al., 1996; Rder et al., 1995, 1998; Stephenson et al., 1998). PCR-based SSR markers are efficient and give more intraspecific polymorphism in wheat than does the restriction fragment length polymorphism (RFLP) method (Plaschke et al., 1995; Röder et al., 1995). SSRs can be utilized on a small scale in breeding programs with only a small investment in a thermocycler and a few gel electrophoresis units. However, to realize the full potential of marker-based selection, which is to follow traits in populations more effectively, a researcher must be able to isolate DNA efficiently from large numbers of samples. The DNA must be of high quality and stable during storage. Thus, the goal of this research was to develop a low cost, high-throughput DNA extraction procedure that eliminates the need for greenhouse space and isolates DNA of substantial quality for use in repeatable PCR reactions.

USDA-ARS Plant Science and Entomology Unit, 4008 Throckmorton Hall, Manhattan, KS 66506. Mention of trademark or proprietary product does not constitute a guarantee or warranty by the USDA and does not imply its approval over other suitable products. Received 17 Dec. 2001. Contribution no. 02-186-J of the Kansas Agricultural Experiment Station. *Corresponding author (jpf@alfalfa.ksu.edu).

Published in Crop Sci. 42:2088–2091 (2002).

MATERIALS AND METHODS

Seedling Germination

The parental lines of the International Triticeae Mapping Initiative (ITMI) wheat population 'Opata' and 'W-7984' (Van Deynze et al., 1995) were used in this study. Seeds were germinated in 8-well, rectangular tissue culture dishes (Nalgene Nunc International, Rochester, NY) containing germination paper (2.5 × 3 cm), 10 seeds per well, and water (Fig. 1). Plates were placed in the dark at 25°C for 24 h, followed by a germination period of 72 h with a 16-h day length at 27°C. One set of tissue was extracted immediately. A second set of tissue was harvested and stored in a 96-well plate at −20°C without buffer. The DNA was extracted from this tissue after 30 d of storage.

DNA Extraction

From the germinated seedling and coleoptile, 1.5 cm of tissue, approximately 0.02 g wet weight total, was harvested from three seedlings. Tissue was placed into a 96-well, flat-bottom tissue culture plate (Nalge Nunc International) on ice. Directly to the tissue, 40 μL of 0.25 *M* NaOH was added to each well plate. The plate was placed in a 95°C water bath for 1 min. Three different maceration techniques were compared. A 0.5-mL pellet pestle (VWR Scientific Products, South Plainfield, NJ) was used to grind individually 96 tissue samples by hand pressure. To macerate 96 samples at one time, a 96-solid-pin replicator (V & P Scientific, San Diego, CA) with hand force was utilized. Finally, a Matrix Mill (Harvester Technology, Inc., Lansing, NY) was used to macerate the tissue mechanically for 1, 3, 5, 7, and 10-min intervals. To each well, 130 μL of 0.1 *M* Tris-HCl, pH 8.0, was added. Plates were centrifuged at 2250 × *g* for 10 min, and then 150 μL of supernatant was removed and placed into a 0.2-mL conical-bottomed, 96-well PCR plate. The DNA was precipitated for purification with 15 μL of 3 *M* NaOAc, pH 5.2, and 120 μL of 100% isopropanol. Samples were then placed at −80°C for 1 h. Plates were centrifuged at 2250 × *g* for 30 min to pellet the DNA. Supernatant was removed by gently tapping the plate on paper towels and centrifuging the plate upside down at 1200 × *g* for 1 min. The pellet was washed with 200 μL of 700 mL/L EtOH/H_2O and again centrifuged at 2250 × *g* for 20 min. Ethanol was removed by centrifuging the plate upside down at 1200 × *g* for 2 min. The DNA was then resuspended in 20 μL of 1× TE (10 m*M* Tris-HCl, 1 m*M* EDTA) buffer, pH 8.0. The DNA was stored at −20°C for 30 d. The amount of DNA in each extraction was quantified with a GeneQuant Pro spectrophotometer (Pharmacia Biotech, Cambridge, MA). Sampling and processing of the tissue were replicated three times.

PCR Amplification

Quality of the DNA was tested by means of wheat microsatellite primers *gwm493* and *gwm533* (Röder, et al., 1998). Forward primers were 5′ labeled with FAM and NED (Applied Biosystems, Foster City, CA). Amplicons were obtained by a 25-μL reaction consisting of 2 μL of DNA extract (<0.1–0.26 μg/μL), 500 pmole forward and reverse primer, 0.25 m*M*

Fig. 1. Four-day-old germinating wheat seedlings in an 8-well culture plate.

dNTP (Sigma, St. Louis, MO), 1× PCR Buffer without $MgCl_2$ (Sigma), 2.5 m*M* $MgCl_2$ (Sigma), and 1.25 units *Taq* DNA polymerase (Sigma). Cycling for PCR consisted of an initial 3 min denaturing step at 94°C followed by 35 cycles of denaturing (94°C) for 1 min, primer annealing (60°C) for 1 min, extension (72°C) for 2 min, and a final extension of 10 min at 72°C.

Electrophoresis and Fragment Analysis

The PCR amplification was verified by gel electrophoresis at 50 V for 4 h in a 1× TBE (45 m*M* Tris-borate, 1 m*M* EDTA) (Sambrook and Russell, 2001), 2.3% (w/v) SFR Agarose (Amresco, Solon, OH) gel. Following verification of the PCR product, fragments were resolved with an ABI 3100 Prism Genetic Analyzer (Applied Biosystems) with 36-cm capillaries. Genescan and Genotyper analysis software (Applied Biosystems) were used to analyze the data.

RESULTS AND DISCUSSION

Germination Culture Dishes

This protocol was developed to take advantage of the ability to extract DNA from germinated seedlings, which avoids greenhouse use and provides a quick turnaround from seed to DNA. Germination dishes (Fig. 1) were evaluated for ease of use and space utilization. Eight-well rectangular tissue culture dishes were chosen for several reasons. Seeds can be germinated on filter paper to produce sufficient tissue for numerous extractions. The extraction is not completely destructive, and the seedlings of interest can be grown later in soil for further study, progeny testing, or to obtain larger quantities of DNA. Tissue that is isolated can be easily transferred to a 96-well format, a standard for laboratory automation. Eight-well dishes were space efficient and allowed for numerous plates to be used in minimal growth chamber space. Finally, the trays can be reused. The culture plates are large enough to be sufficiently cleaned and reused.

Sample Processing and DNA Quality

We have modified the wheat DNA extraction procedure of Dayteg et al. (1998). In optimizing the procedure, we addressed the limiting issues of tissue maceration and DNA quality. Different maceration techniques were compared. Each individual sample of plant tissue was initially ground with a hand-held pestle. A 96-pin replicator was also used to macerate 96 samples at once. Neither hand-grinding techniques provided sufficient DNA quantities to amplify a PCR product (data not shown).

The Matrix mill, a device that utilizes small stainless steel cylinders placed into each of the 96 wells, was another alternative used for tissue maceration. Magnets within the device drive the cylinders in the wells, macerating the plant tissue. Maceration times of 1, 3, 5, 7, and 10 min were compared and DNA concentrations were measured. Concentrations of DNA varied within and

Table 1. The DNA concentration means ± S.E.M., *n* = 3 and 260/280 absorbance ratios from all maceration times of both fresh and frozen wheat tissue.

	Mean		260/280 Ratio	
	Fresh tissue	Frozen tissue	Fresh tissue	Frozen tissue
	μg/μL			
W-7984				
1 min	0.04 ± 0.051	0.01 ± 0.100	1.58 ± 0.169	1.11 ± 0.086
3 min	0.10 ± 0.095	0.01 ± 0.000	1.42 ± 0.183	1.18 ± 0.026
5 min	0.05 ± 0.050	0.26 ± 0.115	1.35 ± 0.205	1.13 ± 0.011
7 min	0.10 ± 0.000	0.10 ± 0.000	1.58 ± 0.105	1.17 ± 0.115
10 min	0.20 ± 0.100	0.13 ± 0.057	1.53 ± 0.098	1.23 ± 0.072
Opata				
1 min	0.13 ± 0.057	0.10 ± 0.100	1.42 ± 0.166	1.14 ± 0.020
3 min	0.13 ± 0.115	0.20 ± 0.264	1.43 ± 0.042	1.24 ± 0.127
5 min	0.02 ± 0.026	0.00 ± 0.000	1.42 ± 0.056	n/a
7 min	0.04 ± 0.052	0.06 ± 0.057	1.35 ± 0.028	1.13 ± 0.115
10 min	0.04 ± 0.048	0.08 ± 0.028	1.48 ± 0.028	1.25 ± 0.014

across maceration times (Table 1). On the basis of DNA concentration, no distinct maceration time was superior. Utilization of the Matrix Mill when compared with hand techniques increased the amount of DNA isolated to a detectable level (as high as 0.26 μg/ L) as well as reducing the time to grind tissue samples from ~1 min per sample to 7 to 10 min per 96 samples.

Maceration times of 7 to 10 min were chosen to broaden the utility of the protocol for use on tissues of different ages as well as for fresh or frozen tissue. Providing DNA of sufficient quality for consistent amplification is a critical issue in regards to effective marker-assisted selection. The DNA extracts from 1-, 3-, 5-, 7-, and 10-min grinding treatments were used for PCR amplification. The PCR amplification experiment tested four treatment and tissue type combinations, including stored DNA isolated from fresh tissue, fresh DNA isolated from fresh tissue, stored DNA isolated from frozen tissue, and fresh DNA isolated from frozen tissue. The CTAB (hexadecyltrimethylammonium bromide)-extracted DNA was used as a quality comparison. The microsatellite markers and wheat lines were chosen because the expected bands were well characterized (Röder et al., 1998). Agarose gels were used to confirm the presence of PCR product, and the products were analyzed by means of capillary electrophoresis. The W-7984 fragments of *gwm493* were 170 base pairs (bp) and *gwm533* were 116 and 166 bp in size for fresh, frozen, and CTAB-extracted DNA. Opata fragments were 137 bp (*gwm493*) and 113 and 166 bp (*gwm533*), respectively, for all treatments (Fig. 2A and B). There were no apparent fragment size differences among the DNA extraction procedures. Also, fresh or frozen tissue samples provided DNA of equal quality after a rapid DNA extraction and 30 d of storage at −20°C.

Efficiency and Costs

The most important issue in regards to high-throughput, marker-assisted selection is efficient use of time

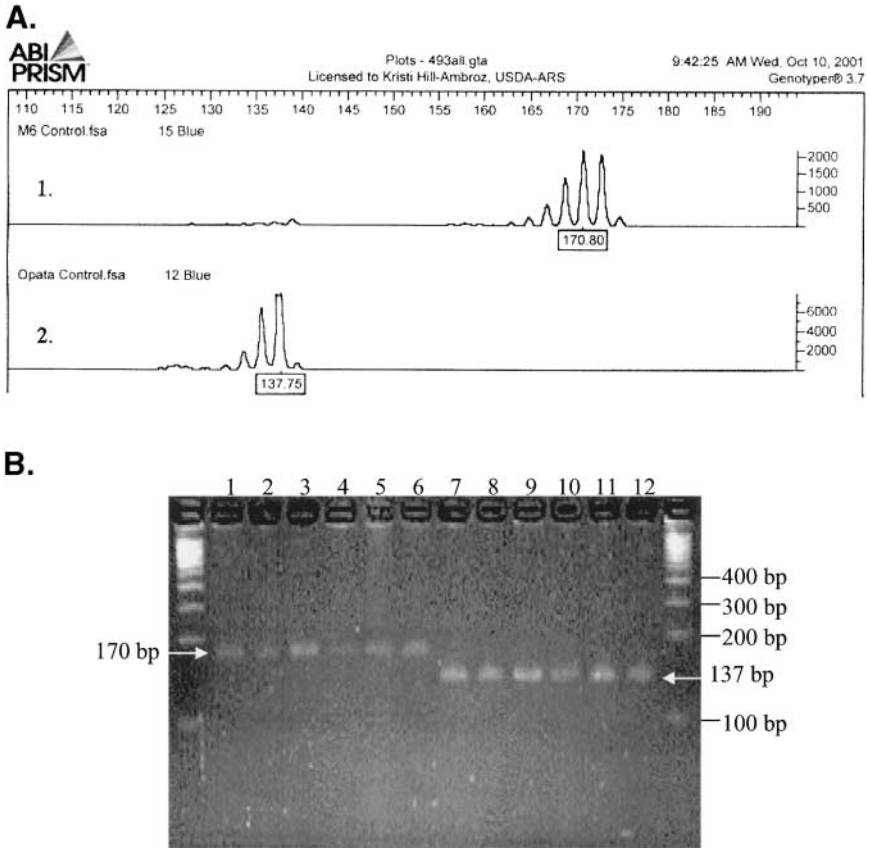

Fig. 2. A) Electropherograms generated from ABI 3100 Prism genetic analyzer. 1—Marker gwm493-W-7984 control CTAB extraction. 2—Marker gwm493 - Opata control CTAB extraction. B) 2.3% SFR gel electrophoresis of rapid DNA extraction amplification with gwm493. 1—W-7984, 1 min. maceration; 2—W-7984, 3 min. maceration; 3—W-7984, 5 min. maceration; 4—W-7984, 7 min. maceration; 5—W-7984, 10 min maceration; 6—W-7984, CTAB control; 7—Opata, 1 min. maceration; 8—Opata, 3 min. maceration; 9—Opata, 5 min. maceration; 10—Opata, 7 min. maceration; 11—Opata, 10 min. maceration; 12—Opata, CTAB control.

and actual costs of materials. Each step of the procedure was evaluated for the chemicals used and the time allotted. The rapid extraction protocol allows for approximately 960 DNA extractions to be done in an 8-h workday. In comparison, a standard CTAB extraction procedure (Murray and Thompson, 1980) can extract only 96 samples in two, 8-h workdays. Estimating labor costs at $10 per hour, isolation of 96 samples will cost approximately $8 for the rapid extraction method and $160 for the CTAB DNA isolation method. Chemical costs are estimated at $0.50 for rapid extraction and $6.72 for CTAB extraction. Plastics are approximately $11 for rapid extraction and $25.82 for CTAB extraction. The total cost for 96 DNA samples is estimated to be $19.50 for rapid extraction and $192.54 for CTAB extraction. Considering an average of 2 μg DNA isolated from each rapid extraction and 10 μg from CTAB extraction, the cost of a rapid extraction becomes $0.10 per μg DNA and CTAB extraction $0.20 per μg DNA. The amount of DNA obtained from the rapid method is much less than from the CTAB method and is a significant limitation to the protocol. Nonetheless, enough DNA is extracted for PCR analysis, and the efficiency allows for large numbers of samples to be processed. This allows breeding programs to utilize marker-assisted selection more efficiently and cost effectively than previous methods.

CONCLUSIONS

Modification of the Dayteg et al. (1998) DNA extraction protocol has provided a high-throughput method to extract a large number of wheat tissue samples efficiently in a short period of time at minimal cost. An impressive 960 DNA extractions can be achieved in a single day. Exposure to hazardous chemicals, such as chloroform, CTAB, and β-mercaptoethanol, is avoided. With isopropanol–ethanol precipitation the need for expensive cleanup kits is eliminated and the stability of DNA samples in storage is improved. This method also increases the reliability and repeatability of the PCR reactions. However, only approximately 2 μg of DNA can be isolated per sample, which limits the utility of the DNA to PCR-based marker systems. Some specialized equipment is required for high-throughput DNA extractions, specifically a Matrix Mill or a similar apparatus as well as a centrifuge that accommodates 96-well plates. It needs to reiterated that this protocol was not designed for isolating large quantities of DNA, but for high throughput of samples. The microsatellite data that have been generated from this protocol are reliable and repeatable. This protocol is routinely used in our laboratory for high-throughput microsatellite marker analysis and can be easily modified to utilize different types of plant tissues.

ACKNOWLEDGMENTS

Special thanks are given to Colleen Truman for her work on perfecting this DNA isolation technique, and Tawanna Ross Vardeman for her expertise in CTAB DNA extraction. The authors also thank Drs. Gary Paulsen and Karl Kramer for critically reviewing this manuscript.

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